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SCANNING ELECTRON MICROSCOPE AND INTRAGINGIVAL MICROORGANISMS IN PERIODONTAL DISEASES

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Abstract

During the past two decades there has been an increased understanding of bacterial invasion as a pathogenic mechanism of periodontal diseases. Scanning Electron Microscope (SEM) has played a key role in supporting the idea that bacterial invasion may be another pathogenic mechanism in periodontal disease. This has been due to the fact that SEM has a larger depth of focus and better resolving power than the Light Microscope (LM) and also allows for observation of rather large areas of tissue showing in depth the surface of the sample.

This review deals with information obtained by using SEM as the fundamental method in studying and specifically identifying microorganisms within gingival tissues. New methodology using correlative microscopy for rapidly identifying invasive bacteria of periodontal tissues is discussed.

Key Words: Intragingival Microorganisms, Bacterial Invasion, Scanning Electron Microscopy, Periodontal Disease.

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Introduction

The first Scanning Electron Microscope (SEM) paper on bacterial invasion (BI) of the periodontal tissues was finalized by stating: "The SEM allows excellent visualization of both individual cells and larger populations and may, consequentially, provide additional information about the relationship between bacteria and the host. This preliminary study indicates that a vast spectrum of information remains to be harvested with this remarkable tool." (Saglie, 1977).

Since that time, ten years have passed and a variety of papers have been published on this subject by using SEM. Although it is not possible to differentiate between viable and nonviable cells with SEM, the instrument provides information which may supplement that obtained elsewhere. The main purpose of this review is to gather the information obtained by using SEM as the tool of choice in studying BI of gingival tissue, to discuss its relevance, and to inform about preliminary results of unpublished material.

Historical Background

In 1975, by using extracted teeth having periodontal disease, Saglie et al. (1975) observed under the stereomicroscope that in certain deep pockets the space between the most apically located subgingival plaque and junctional epithelium could be nonexistent. In an attempt to provide an explanation using SEM, Saglie (1977) demonstrated that the most apically located subgingival plaque may invade the junctional epithelium in these cases. The possibility of artifacts was ruled out because of the natural relationship between the bacteria and the host epithelial cells. Later, Saglie and co-workers conducted a number of studies providing additional support for the idea that bacterial invasion of the soft tissue and the bone may be a common finding in advanced periodontitis and juvenile periodontitis in humans. In the majority of these research studies the SEM was the main tool directed to detect the invaders and later to morphologically identify

them. Attachment of bacteria to host cells and topographical relationship between bacteria and gingival diseased tissues were also studied.

Using SEM Saglie et al. (1982 a) reported bacteria within the epithelial wall of deep periodontal pockets in five of eight cases of advanced chronic periodontitis. Observations were made on teeth extracted with a portion of their periodontium attached and on gingival biopsies. Pockets were greater than six mm and extreme bone loss was present. They also reported the presence of bacteria penetrating between the enlarged intracellular spaces of the pocket epithelial surface. Large accumulation of cocci, rods and short filaments on the epithelial side of the basal lamina (BL), were found and it was suggested that the bacteria move toward the BL, where further progress is prevented and accumulation takes place. The penetration of bacteria into the connective tissue took place only in areas where a perforation or interruption of the basement membrane was found. Penetration of cocci, rods, filaments, and spirochetes into the junctional epithelium was also reported, thus confirming previous findings, (Frank, 1980).

Bacteria were found in specific intercellular locations, with a definite pattern of penetration and not a random disarray of bacteria which could correspond to artificial introduction of bacteria during procurement of processing. Leukocytes were sometimes found in connection with invading bacteria. The same authors also described the topography of epithelial surfaces of deep epithelial pockets in humans. They demonstrated the presence of microtopographically distinct areas, suggesting that the pocket wall was constantly changing as the result of the interaction between the host and the bacteria. They described areas of relative quiescence, bacterial accumulation, emergence of leukocytes, leukocyte-bacterial interaction, and epithelial desquamation (Saglie et al 1982 b). The interaction of leukocytes and bacteria inside the gingival tissues in human periodontitis was also studied by using SEM. In the former study, the interaction of leukocyte and bacteria on the surface of the pocket epithelium was documented in various phases of recognition, attachment, and engulfment. Leukocytes were detected coming out from gingival connective tissue blood vessels by diapedesis and were described in the following locations: in peripheral blood vessels adjacent to the pocket epithelium, gingival connective tissue, basement lamina sectioned pocket epithelium, surface of pocket epithelium, and junctional epithelium and cementum surface. Morphologic data suggesting the process of degranulation was also presented. (Saglie et al. 1982 d).

The first attempts to specifically identify invasive microorganisms in periodontal disease were made by the UCLA group using a combined methodology which included culture SEM, Transmission Electron Microscope (TEM), immunocytochemistry, and stained histological sections. Spirochetes were identified by using SEM and TEM; mycoplasma by using SEM, TEM, specific stains, and specific culture medium; Actinobacillus actinomycetemcomitans by using immunocytochemical technique, TEM (pop-off) technique (Bretschneider et al., 1981) and cultural methods; and Capnocytophaga sputigena by using immunocytochemical technique. Gram-negative bacteria were identified by TEM and Gram stain. (Saglie et al. 1982 c).

In a SEM and TEM study of tissue-invading microorganisms in localized juvenile periodontitis (Carranza et al. 1983) the invasion of bacteria (mainly gram-negative fusiform, coccobacilli, and spirochetes) into the gingival tissue and along resorbing bone was documented in a 15 year-old patient. Bacteria were described as penetrating through tissue clefts and ulcerated areas, invading the epithelium and the connective tissue and reaching the surface of bone. Mycoplasma were found also to invade in some areas. They reported a clear tissue-bacteria relationship shown by the presence of microconcavities containing bacteria, suggesting an active infiltration process. Some microorganisms were seen in lacunae of the superficial alveolar bone.

SEM, which may be considered the tool of choice in identifying mycoplasm, was successfully used to detect mycoplasma invading the periodontium of a 15 year-old female patient. (Newman et al. 1984).

Being aware that the technique available for detecting bacteria in histological sections is time consuming, Saglie and coworkers initiated a series of experiments in order to develop a method for a rapid in situ identification of bacteria in one tissue section by using Light Microscope (LM), SEM, and TEM. Preliminary results with this new technique showed that this method may be a simple, rapid, and definitive method for bacterial identification in a large number of section samples. (Saglie et al. 1985 b).

Recently, in a series of studies, some of which are not yet published, this correlative microscopic method has been utilized to substantiate the bacterial nature of Gram-stained particles in gingival diseased histological tissue sections: from our laboratories, the bacterial presence in the oral epithelium has been confirmed, (Saglie et al. 1985 a, 1986, 1987 b,c,d, Pertuiset et al., 1987). BI in the initiation of gingival inflammation has been suggested by demonstrating a correlation between the number of intragingival microorganisms and the severity of gingival inflammation during experimental gingivitis in humans (Saglie et al. 1987 b). In a study of Saglie and co-workers, the mononuclear infiltrates in consecutive histological sections to gram-stained and peroxidase stained sections showing bacterial presence were characterized. Active and inactive sites were identified according to the quality of the infiltrate, which was identified by using

monoclonal antibodies. They concluded from this study that suspicious pathogens having the capacity to be invasive, such as A. actinomycetemcomitans and B. gingivalis, may prepare in situ the production of an inflammatory infiltrate which is deleterious for the host, (Saglie et al. 1988 a).

The first report of yeast within diseased gingival tissue in localized juvenile periodontitis using SEM has been reported this year by Gonzalez et al. (1987).

To testify the hypothesis previously expressed by Allenspach-Petrsilka and Guggenheim in (1982), that bacterial invasion is linked to disease activity, Saglie et al. (1988 d) initiated the following investigations: By using the loss of attachment as a parameter they monitored 20 patients per week, all of whom had been previously selected by having radiographic evidence of advanced bone loss and pocket depth of more than five mm. When disease activity was detected surgery was performed and the tissue fixed and processed for several procedures oriented to identify the intragingival microorganisms. They used, as control, non-active sites with the similar pocket depth from the same patient, Preliminary results have shown, by t-test, that active sites showed a significantly higher number of bacteria in the connective tissue when compared with non-active sites. The specific identification of the invaders and the identification of the inflammatory infiltrate by using monoclonal antibody in consecutive histological sections, also part of the study, may shed light upon bacterial invasion as being a very suspicious etipathogenic mechanism of tissue destruction.

Discussion

The SEM has been a powerful instrument with which to study BI of periodontal tissues. Compared to other microscopic techniques, it permits the examination of the largest areas of surface at a resolution of better than 10 mm. It is particularly well-suited for the study of the detailed spatial distribution of invasive bacterial groups on top of periodontal tissues. But the search of an invasive bacteria within tissue with SEM may be a time-consuming procedure if one does not know the specific site of invasion or how deep the colonies have invaded the tissues (Saglie & Elbaz, 1983).

A good tool for precisely identifying bacteria in diseased gingival tissues is the combination of LM, SEM, and TEM for studying the same histological section. Even the bacterial nature of the gram-stained material can be considered doubtful, as little information is provided by a gram-stained LM section (Fig. 1A). However, various serial sections with a large surface each can be quickly scanned for the presence of bacteria or bacteria-like material (Fig. 1B).

It is possible to achieve larger magnifications with the SEM, which also provides its large depth of focus. As a result, one can clearly recognize the shape, size, and number of those considered to be bacteria under the LM. Using SEM to find bacteria in the tissue can prove a long and tedious procedure. Considerable time and effort can be saved by using this method, as the gram-stained LM section can direct the observer rapidly to the areas of interest.

In the SEM, spirochetes, filamentous bacteria, and rod-shaped bacteria can be positively identified as such, requiring no further TEM (Figs. 2-3-4). Despite this fact, however, several granular shaped particles may resemble cocci forms, making necessary an additional TEM positive identification. Granules of mast cells, mitochondria, and other intracellular organelles and granules can be included among those particles resembling cocci forms.

After sectioning and staining the SEM sample for TEM, the fine structure characteristics of these cocci particles give us the final proof of their bacterial nature. On many occasions, ultrastructural details of bacteria and host tissues are not very well preserved. The use of rather powerful organic solvents (xylene), the heat for paraffin embedding (about 56 degrees Celsius), and the crystallization of the paraffin, can cause some tissue damage. No artifacts are considered to be produced by the critical point drying for SEM. Ultrastructural details are generally preserved enough for the purpose of bacterial identification, despite the procedures for LM preparation.

We showed that a reliable technique for bacterial identification is gram-staining; most of the gram positive and gram negative stained particles proved to be bacteria. Nevertheless, to determine bacterial morphotypes precisely, SEM after LM is an important tool. There were cases in which the classical bacterial morphotypes under SEM were not demonstrated by gram-stained material. However, because bacterial components or disintegrated bacterial fractions take gram-stain (Pekovic and Fillery, 1984), we cannot completely disregard the possibility of their bacterial nature. It has been shown that keratinized epithelium, epithelial cells, and tissue structures surrounding infiltrated bacteria and antigens can also be stained by Gram stain (Pekovic and Fillery, 1984, Saglie et al. 1985 a,b). So, in most cases, SEM may be a useful tool for final identification. We also achieved good results when, recently, we used this same technique with sections stained with antibacterial antibodies and peroxidae (Saglie et al. 1986).

To reduce the possible artifacts introduced mainly during tissue preparation for LM, precautions can be taken, although it was not necessary to achieve our goal. For dehydration and clearing, mild organic solvents can be used, and tissue sections, while being prepared for, or studied under, LM, can be kept "wet". Other authors studying blood smears successfully under LM and SEM (Wetzel et al. 1973) have successfully used this last

approach. It was recently described by Kondo (1984) that, instead of paraffin, polyethylene glycol can also be used as embedding medium and removed after sectioning for LM.

The study of the same cells under more than one type of microscope has been reported by previous publications. Cell suspensions, such as blood cells (Wetzel et al. 1973), bone marrow cells, tumor cells in suspensions (Bahr et al. 1976; Takenaga et al. 1977), or cell monolayers grown in cultures (Thornthwaite et al. 1976; Kumon et al. 1983), were involved in all LM studies followed by SEM. Most researchers scratched the coverslip or glass slide with diamond markers or marked the glass slide or gridded patterns to relocate the same cells under the SEM. To detect microinvasive carcinomas (Murphy et al. 1973), post-SEM histological LM has been used as well. TEM following LM of semithin epoxy sections (Campbell & Hermans 1972), and TEM sectioning of previously paraffin-embedded tissue ultrastructure artifacts (Kobernick & Thomas 1970), have been reported. However, this is the first report, to the best of our knowledge, of successful study of one LM paraffin section under SEM and TEM after LM staining. Recently, we expanded the usefulness of this technique by using this same procedure for SEM and TEM observation of previously immunoperoxidase stained bacteria. Furthermore, the sectioning for TEM of a three m thick piece of tissue is the only stage requiring special care and/or skill.

Conclusions

1. SEM is considered the tool of choice to identify, undoubtedly, some microorganisms within tissues, such as spirochetes and mycoplasma.
2. SEM after LM is an important tool to determine bacterial morphotypes precisely.
3. SEM is an ideal apparatus to be used in the study of topographical relationship between invasive periodontal pathogens and host gingival tisses.

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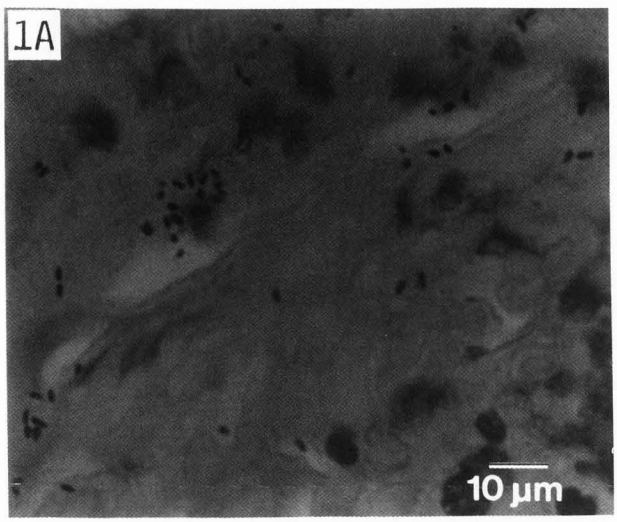

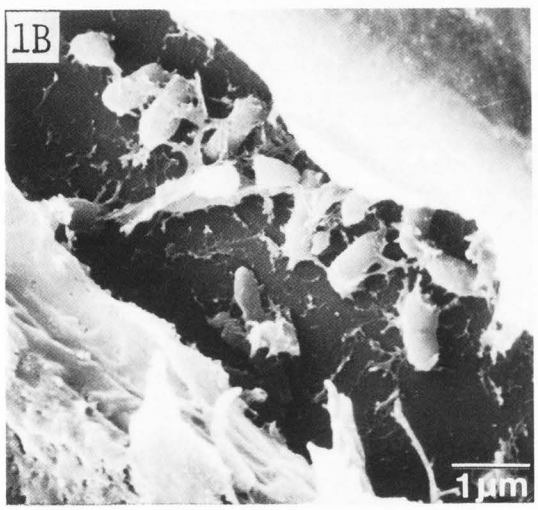

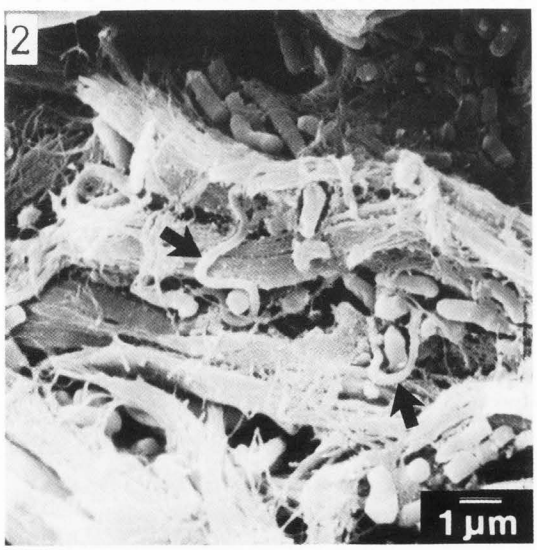

Fig. 2. Intragingival connective tissue bacteria identified under the SEM by typical morphology: spirochetes (arrow).

Fig. 1A). LM micrograph of a gram-stained section showing collagen fibers, several cells and gram-stained bacteria-like particles. Fig. 1B). The same gram-stained section of Fig. 1A after preparation for SEM observations (Saglie et al., 1986). Bacterial morphology can be recognized (rod-shaped bacteria).

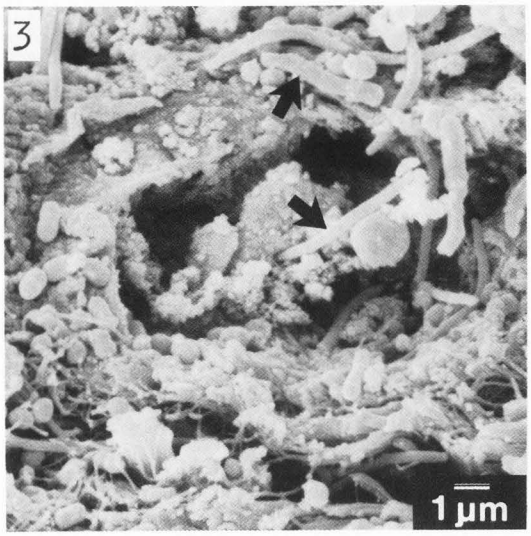

Fig. 3. Filaments (arrows) and rod-shaped intragingival bacteria (Fig. 1B) are with spirochetes (Fig. 2) easily identified under SEM (Fig. 2).

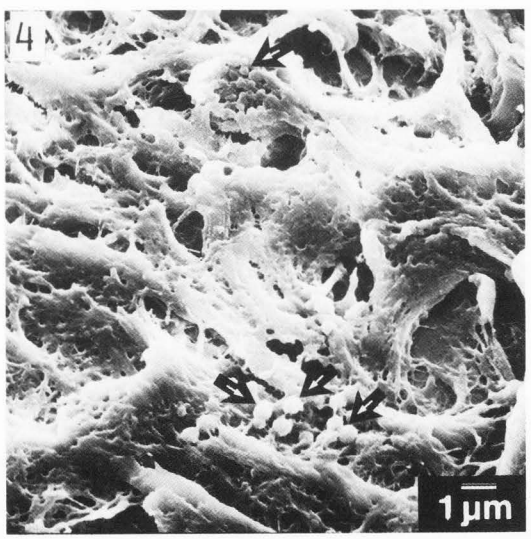

Fig. 4. When coccoid-shaped particles are found under SEM as is shown in this electron micrograph their bacterial nature becomes doubtful (arrows). These areas should be inscribed, embedded and sectioned for TEM (Saglie et al 1985). In this way the bacterial nature of the suspicious particles could be identified beyond doubt.

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Discussion with Reviewers

A. Carrassi: How can you differentiate an intragingival bacterial invasion from passively introduced microorganisms?

Author: I differentiate an intragingival bacterial invasion from passively introduced microorganisms by:

1) Pattern of bacterial invasion (per example bacteria in "chains" between intercellular space).

2) Intracellular located bacteria.

3) Biopsies of normal gingiva used as control did not contain bacteria.

4) Homogeneous colonies of bacteria within connective tissue.

S.H. Ashrafi: How would you recognize various types of bacteria invading pocket wall epithelium by using SEM?

Author: I recognize them primarily by bacterial morphology and size, pattern of colonization and topographical relationnship with host tissue. For example mycoplasma. They were identified penetrating the epithelium and connective tissue under the SEM by their small size (0.2 - 0.5 μm) polymorphism/hemadsorption to host red blood cells, hemagglutination, hemolysis and the ability to attach to other cell surfaces such as epithelial cells, leukocytes and fibroblasts.